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AGE-RELATED CHANGES OF CANCELLOUS AND CORTICAL BONE HISTOMORPHOMETRY IN FEMALE SPRAGUE-DAWLEY RATS

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Abstract

Double fluorescent labeled virgin female Sprague-Dawley rats were serial sacrificed between 3 and 16 months of age to characterize age-related skeletal changes. Histomorphometric analyses were performed on microradiographs and 20 μm undecalcified longitudinal sections of the proximal tibia and fourth lumbar vertebral body and cross sections of tibial shafts. The most consistent age-related change among all test bone sites is a decrease in skeletal cellular based metabolic activities, including reductions in both recruitment and functional activity for osteoclasts and osteoblasts. Age-related cancellous bone loss with a discontinued trabecular structure was observed in proximal tibial metaphysis. Although cancellous bone mass in the lumbar vertebral body does not change with increasing age, there was a significant increase in trabecular width. Age-related cortical bone change involves a redistribution of equal amounts of bone mass from the cortical-endosteal region to the subperiosteal region, leading to an expansion in both subperiosteal and marrow areas while cortical areas remain unchanged.

Key Words: aging, cancellous bone, cortical bone, bone mass and architecture, longitudinal and radial growth, static and dynamic histomorphometry.

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Introduction

The rat is widely used to test the effects of drugs, hormones, nutritional and growth factors, and diseases on the structure and composition of bone. Some characterization of the age-related skeletal changes have been performed, but it is incomplete [14, 17, 18, 19, 29, 35, 36, 42, 43]. Characterizing these changes would be useful. Such information will further our understanding of the rat skeletal development and maintenance; this will enhance our use of the rat in the prevention and treatment of metabolic bone diseases. Without this information, it would be impossible either to properly measure or interpret experimental results. Therefore, the purpose of the current study is to characterize the age-related skeletal histomorphometric changes in Sprague-Dawley female rats, including the amount of cancellous and cortical bone mass, their structural architecture, and rate of turnover (modeling and remodeling).

Materials and Methods

Experimental Design

Sixty-four virgin female Sprague-Dawley rats (Simonsen Laboratories, Inc., Gilroy, CA) were serial sacrificed at 3, 7, 8, 9, 10, 12, 14 and 16 months of age. Before sacrifice, animals were housed singly in 21 x 32 x 20 cm cages and maintained on a 12 hour/12 hour light-dark cycle. They were allowed free access to water and a pelleted commercial diet (Wayne Rodent Blox 8604, Teklad Premier, Madison, WI) containing 1.46% calcium, 0.99% phosphorus and 4.96 I.U. Vitamin D_3/g. All animals received subcutaneous injections of tetracycline (25 mg/kg); achromycin tetracycline hydrochloride (Lederle Laboratory, Pearl River, NY) and calcein (10 mg/kg) (Sigma Chemical Co., St. Louis, MO) at 12 and 2 days before sacrifice.

Autopsy

All rats were sacrificed by cardiac puncture under an anesthesia consisting of ketamine hydrochloride and xylazine. 0.3 mm deep frontal sections of the tibial tuberosity of the proximal tibia and 3 mm of the left border of the fourth lumbar vertebral body along with the

Table 1a. Measurements of proximal tibial metaphyseal and lumbar vertebral body cancellous bone.

Measured Parameters	Abbreviations	Definitions	Unit
Trabecular area	Tb.Ar	Total cancellous bone area within total area	mm^2
Trabecular perimeter	Tb.Pm	The perimeter of Tb.Ar	mm
Single label perimeter	sL.Pm	The length of trabecular surface labeled with tetracycline or calcein	mm
Double label perimeter	dL.Pm	The length of trabecular surface labeled with both tetracycline and calcein	mm
Interlabel width	Ir.L.Wi	The distance between tetracycline and calcein labels	μm
Interlabel width (growth)	Ir.L.Wi.-G	The distance between tetracycline and calcein labels in growth plate metaphyseal junction region	μm
Eroded perimeter	E.Pm	The length of trabecular surface with Howship's lacuna	μm
Osteoid perimeter	O.Pm	The length of trabecular surface covered with osteoid	mm
Wall width	W.Wi	THe distance between reversal line and trabecular surface	μm

Table 1b. Calculations of proximal tibial metaphyseal and lumbar vertebral body cancellous bone.

Calculated Parameters	Abbreviations	Formulae	Units
Percent trabecular area	%Tb.Ar	Tb.Ar/T.Ar	%
Trabecular width	Tb.Wi	(2000/1.199) x Tb.Ar/Tb.Pm	μm
Trabecular number	Tb.N	1.199/2 x Tb.Pm/T.Ar	#/mm
Trabecular separation	Tb.Sp	(2000 x 1.199) x (T.Ar - Tb.Ar)/Tb.Pm	μm
Percent eroded perimeter	%E.Pm	E.Pm/Tb.Pm x 100	%
Percent labeled perimeter	%L.Pm	(dL.Pm + sL.Pm/2)/Tb.Pm x 100	%
Mineral apposition rate	MAR	Ir.L.Wi/Interval	$\mu m/d$
Bone formation rates (bone area based)	BFR/BV	(dL.Pm + sL.Pm/2) x MAR/Tb.Ar x 365 x 100	%/y
Longitudinal growth rate	LGR	Ir.L.Wi-G/Interval	$\mu m/d$
Formation period	F.P	W.Wi/MAR	days
Resorption period	R.P	F.P x E.Pm/O.Pm	days
Remodeling period	Rm.P	F.P + R.P	days
Longitudinal growth rate	LGR	Ir.L.Wi-G/Interval	$\mu m/d$

pedicles were sawed using a low-speed metallurgical saw to expose the marrow cavity for better fixation. The bone specimens were immersion fixed in 10% phosphate buffered formalin (pH 7.2) for 24 hours, then transferred to 70% ethanol.

Bone specimen preparation

After fixation, bone specimens were dehydrated in graded concentrations of ethanol, defatted in acetone, then embedded in methyl methacrylate (Eastman Organic Chemicals, Rochester, NY). Undecalcified longitudinal sections of the proximal tibia and the fourth lumbar vertebral body (2 sections for each site), and cross sections of the tibial shaft (3 sections per site) were sawed to 230 μm in thickness using a low-speed metallurgical saw, then ground to 100 μm using a precision lapping machine (Maruto Co., Tokyo, Japan) for microradiography

Table 2a. Histomorphometric measurements of tibial shaft cortical bone.

Measured Parameters	Abbreviations	Units
Total tissue area	T.Ar	mm^2
Periosteal perimeter	P.Pm	mm
Marrow area	Ma.Ar	mm^2
Endosteal perimeter	E.Pm	mm
Periosteal Surface		
Single labeled perimeter	P-sL.Pm	mm
Double labeled perimeter	P-dL.Pm	mm
Interlabel width	P-IrL.Wi	μm
Endosteal Surface		
Single labeled perimeter	E-sL.Pm	mm
Double labeled perimeter	E-dL.Pm	mm
Interlabel width	E-IrL.Wi	μm

Table 2b. Histomorphometric calculations of tibial shaft cortical bone.

Measured Parameters	Abbreviations	Formulae	Units
Cortical area	Ct.Ar	T.Ar - Ma.Ar	mm^2
Percent cortical area	%Ct.Ar	Ct.Ar/T.Ar x 100	%
Percent marrow area	%Ma.Ar	Ma.Ar/T.Ar x 100	%
Periosteal Surface			
Mineral apposition rate	P-MAR	P-Irl.Wi/Interval	$\mu m/d$
Labeled perimeter	P-L.Pm	P-sL.Pm/2 + P-dL.Pm	mm
Percent labeled perimeter	P-%L.Pm	P-L.Pm/P.Pm x 100	%
Bone formation rate (surface based)	P-BFR	P-L.Pm x P-MAR/P.Pm x 100	$\mu m/d$
Endosteal Surface			
Mineral apposition rate	E-MAR	E-IrL.Wi/Interval	$\mu m/d$
Labeled perimeter	E-dL.Pm	E-sL.Pm/2 + E-dL.Pm	mm
Percent labeled perimeter	E-%L.Pm	E-L.Pm/E.Pm x 100	%
Bone formation rate (surface based)	E-BFR	E-L.Pm x E-MAR/E.Pm x 100	$\mu m/d$

on Kodak spectroscopic plates (649-0 Eastman Kodak, Rochester, NY). Sections were then mounted on plastic slides using cyanocrylate ester glue (Adhesive 910, Permabond, Englewood, NJ), further ground to thickness of 20 μm, stained with 0.1% Toluidine blue 0, and coverslipped for microscopic analysis [11].

Bone histomorphometry

All sections were analyzed using a digitizing system consisting of a light and epifluorescent microscope with a drawing tube, a graphic pad and an Apple Macintosh computer. Measurements of cancellous bone in the proximal tibia were taken from a 6 mm^2 (2 x 3) area in the central region beginning at 1 mm distal to the growth plate, and that in the lumbar vertebral body were taken from the entire metaphyseal region with a 0.4 mm margin from cortical bone. For both sites, measurements included: total tissue area, trabecular area and perimeter, eroded perimeter, single and double fluorescent labeled perimeters, and interlabeling width produced by

Table 3. Age related changes in proximal tibial metaphyseal cancellous bone histomorphometry.† See Table 1a and 1b for definitions of the abbreviations.

Age (mos.)	LGR (μm/d)	Tb.Ar (%)	Tb.Wi (μm)	Tb.N (#/mm)	Tb.Sp (μm)	L.Pm (%)	MAR (μm/d)	BFR (%/yr)	E.Pm (%)
K-W H test‡	0.0001	0.0001	0.0002	0.0001	0.0001	0.0404	0.0001	0.0001	0.0020
3	69.06	24.40	45.87	5.36	141.53	11.38	1.75	266.25	4.57
SD	11.75	2.78	7.67	0.39	9.60	3.30	0.43	99.12	0.66
7	10.62	23.49	47.35	4.97	160.64	9.55	1.12	144.07	6.31
SD	3.63	4.72	3.55	0.97	40.34	2.81	0.24	77.97	0.75
8	5.36	22.09	45.77	4.81	166.19	8.92	1.08	126.80	6.04
SD	1.39	4.10	3.66	0.67	34.79	0.99	0.20	17.49	0.94
9	3.74	17.68	40.71	4.35	189.39	8.59	1.13	144.55	5.51
SD	0.90	1.00	3.07	0.15	6.17	1.67	0.21	34.09	0.85
10	2.43	18.00	41.02	4.39	186.74	8.11	1.03	124.65	5.49
SD	0.73	0.86	2.63	0.12	4.32	1.35	0.21	36.67	1.50
12	1.74	14.45	38.52	3.74	231.63	7.46	0.83	99.05	5.13
SD	0.72	2.26	2.80	0.39	29.75	1.64	0.31	44.68	1.67
14	1.32	14.97	45.39	3.31	257.59	7.18	0.64	58.75	3.60
SD	0.75	1.02	4.12	0.14	11.10	2.46	0.17	17.51	0.95
16	1.08	14.72	43.49	3.39	252.04	7.10	0.58	56.77	3.82
SD	0.67	1.13	3.23	0.09	7.77	1.85	0.07	14.49	1.19
Linear regression analysis (age as independent parameter)									
Intercept	51.87	27.83	46.00	6.11	92.65	11.90	1.89	279.57	6.526
Slope	-4.38	-0.98	-0.33	-0.19	10.93	-0.36	-0.09	-15.81	-0.141
SE	0.53	0.11	0.18	0.02	0.93	0.08	0.01	1.92	0.051
r	0.72	0.74	0.23	0.81	0.83	0.52	0.77	0.72	0.333
p	0.0001	0.0001	ns	0.0001	0.0001	0.0001	0.0001	0.0001	0.0070

† Six rats in each group. ‡ Kruskal-Wallis H-test.

linear lamellar bone formation on individual trabecular surface. The above information was used to calculate percent trabecular area, width, number and separation, percent eroded perimeter, percent labeled perimeter, mineral apposition and bone formation rates. In addition, interlabeling widths in the growth plate metaphyseal junction region which produced by longitudinal growth were measured and longitudinal growth rate was calculated for proximal tibia. Osteoid perimeter and wall widths were measured to calculate formation and resorption periods for lumbar vertebral body [10, 24; Table 1].

For the mid-tibial diaphyseal cortical bone, total cross sectional area, cortical area, marrow area, single and double fluorescent labeled perimeter and interlabeling width for both periosteal and endocortical surfaces were measured; percent cortical area, percent labeled perimeter, mineral apposition and bone formation rates for periosteal and endocortical surfaces were calculated [11, 28; Table 2].

Statistical analysis

The statistical difference between various age groups was tested by Kruskal-Wallis H-test [38]. The data was further analyzed as a function of age by linear regression [37].

Results

Proximal tibial metaphyseal cancellous bone

Table 3 summarizes the age-related static and dynamic bone histomorphometric changes in proximal tibial metaphyseal cancellous bone. The rate of longitudinal bone growth decreases rapidly between 3 and 7 months of age; the bone growth rate then continues to decline slowly between 8 and 11 months, stopping altogether in some rats after they are 12 months old. Maximum or peak cancellous bone mass (percent of trabecular area) accumulates during the first 3 months of life. Rats maintain this peak bone mass until they are 8 months old. At 9 months, age-related cancellous bone loss begins and continues until the rats are 12 months

Table 4. Age related changes in lumbar vertebral body cancellous bone histomorphometry. See Table 1a and 1b for definitions of the abbreviations.

Age (mos.)	Tb.Ar (%)	Tb.Wi (μm)	Tb.N (#/mm)	Tb.Sp (μm)	E.Pm (%)	O.Pm (%)	L.Pm (%)	MAR (μm/d)	BFR (%/yr)	R.P (d)	F.P (d)	Rm.P (d)	W.Wi (μm)
ANOVA	0.977	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
3	23.37	48.63	4.85	232	3.86	8.55	5.43	1.82	113.43	3.78	6.89	10.67	12.59
SD	2.73	3.31	0.38	25	0.80	4.03	1.16	0.16	25.70	2.06	1.25	2.95	2.94
7	24.22	49.75	4.87	229	2.10	4.81	2.80	0.84	26.00	6.73	14.74	21.47	12.22
SD	2.92	4.73	0.11	24	0.66	1.60	0.87	0.13	5.43	1.99	1.26	3.07	1.22
8	24.17	56.77	4.26	256	2.03	4.90	3.58	0.86	33.83	6.43	15.55	21.98	12.82
SD	1.88	5.12	0.25	17	0.50	0.75	0.94	0.17	13.99	1.99	4.57	6.46	1.18
9	23.05	58.23	4.00	281	1.70	4.74	3.28	0.85	29.38	5.95	16.21	22.16	13.62
SD	2.18	5.11	0.33	31	0.12	0.92	0.31	0.06	8.07	1.15	1.62	1.78	0.62
10	24.33	61.54	3.71	292	1.52	3.91	3.20	0.83	25.96	6.90	17.56	24.46	14.48
SD	2.47	6.60	0.24	22	0.15	0.46	0.56	0.07	1.94	1.33	1.87	2.43	1.23
12	23.07	64.04	3.64	311	1.40	3.06	3.15	0.83	26.44	8.81	18.52	27.32	15.30
SD	3.43	6.41	0.45	47	0.16	0.77	0.43	0.06	5.21	1.75	1.49	2.16	0.55
14	22.79	64.72	3.57	317	1.29	2.32	3.12	0.82	25.16	10.91	19.78	30.69	16.20
SD	2.35	5.97	0.26	20	0.20	0.22	0.57	0.06	3.04	0.77	1.34	1.58	0.49
16	23.54	65.62	3.43	324	1.10	2.15	3.01	0.80	22.28	10.94	20.34	31.27	16.24
SD	5.23	5.96	0.68	39	0.20	0.49	0.51	0.05	3.23	3.81	1.28	4.55	1.00
Linear regression analysis (age as independent parameter)													
Intercept	23.93	47.02	5.02	213.97	3.35	7.93	4.52	1.42	79.49	3.18	8.79	11.96	11.38
Slope	-0.08	2.60	-0.22	14.82	-0.32	-0.79	-0.23	-0.10	-8.90	0.96	1.62	2.58	0.63
SE	0.18	0.33	0.02	1.79	0.38	0.11	0.06	0.02	1.60	0.13	0.17	0.25	0.09
r	0.07	0.75	0.80	0.77	0.78	0.72	0.50	0.63	0.64	0.74	0.81	0.84	0.73
p	ns	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

old. Bone mass is then maintained at a low level without further decrease for up to 16 months. Despite the age-related bone loss, trabecular width does not change significantly, but it does display a decreasing trend when bone mass falls between ages 8 and 12 months, and then returns to near previous level when bone mass stabilizes at a low level. However, the significantly decreased trabecular number and increased trabecular separation indicate that the trabeculae are farther apart. With increasing age, decreases in percent of labeled surface, mineral apposition and bone formation rates are observed as early as 7 months of age. These decreases proceed slowly and reach their lowest levels at about 14 months of age. The reduction of eroded perimeter is not observed until rats are 8 months old, and reaches its lowest level at the same time as formation parameters.

Lumbar vertebral body metaphyseal cancellous bone

As listed in Table 4, no age-related change could be detected in cancellous bone mass. However, trabecular width and trabecular separation are increased, and trabecular number decreased, with aging. The bone formation and resorption parameters fall rapidly between 3 and 7 months of age, and remain at the low levels thereafter. With increasing age, wall width is increased and both formation and resorption periods are prolonged.

Mid-tibial diaphyseal cortical bone

Age-related cortical bone changes in tibial diaphysis are listed in Table 5. These changes included continuous increases in total tissue (subperiosteal) area and marrow area, and decreases in the percent of cortical bone area. However, the cortical bone area did not change with aging. Percent labeled surfaces, mineral apposition rates and bone formation rates in both periosteal and endocortical surfaces decreased rapidly between 3 and 8 months of age. Thereafter, periosteal formation parameters remained at low levels and the endocortical mineral apposition rate was not measurable. Only single label surfaces could be observed on the endocortical surface in most 9-month old rats or older rats.

Discussion

In the proximal tibial metaphysis the peak cancellous bone mass accumulates at about 24% of the metaphyseal tissue area and an optimal trabecular architecture develops during the first 3 months. The peak bone mass is maintained without significant changes until 8 months of age. Thereafter, bone mass begins to decrease with aging and reaches a low value of 14% of the metaphyseal tissue area at 12 months of age. The age-related cancellous bone loss in the current study is earlier than that reported by Wronski and his colleagues [42],

in which the sham ovariectomized control rats did not show a reduction until they were 12 months old. Possibly this difference is due to a difference in area sampled between the two laboratories. We excluded the trabeculae near the cortical bone while Wronski and colleagues included them. In the funnel region of the metaphysis, where cortical bone is much thinner than that of the diaphyseal region, the neighboring trabeculae (near the cortical bone) are thicker than centrally located trabecular because they are believed to be needed in supporting mechanical loads. Often, in the osteopenic conditions, these trabeculae survived and demonstrated compensatory thickening. In contrast, most trabeculae located in the central metaphyseal region are thinned or lost. This may also be the case during the development of age-related osteopenia.

The age-related changes in proximal tibial cancellous bone structural indices are similar to those in the human ilium reported by Parfitt *et al.* [27] when expressed by linear regression. Significantly decreased trabecular number and insignificantly decreased trabecular width with aging indicate that the normal loss of trabecular bone with age occurs predominantly by a process that removes entire structural elements of bone, leaving those that remain more widely separated but only slightly reduced in thickness, thus transforming the mainly continuous trabecular network characteristic of a younger subject into the mainly decreasing interconnectedness of the normal trabecular bone characteristic of the elderly [27].

Interestingly, no age-related cancellous bone loss is detected in the metaphysis of the lumbar vertebral body by either analysis of variance or linear regression. A similar phenomenon was found by Wronski *et al.* [43] in sham ovariectomized control rats. However, we further observe that with increasing age, trabecular width is significantly increased and trabecular number is significantly decreased, suggesting that the structure of cancellous bone is altered in a way that some trabeculae disappear while others are thickened. In human vertebral cancellous bone, age-related structural changes were revealed by radiographic [34, 40] and histological [1, 2, 3] studies. The studies show that horizontal trabeculae were preferentially lost while the remaining vertical trabecular plates were thickened. Thus far, we have not ascertained if this is also true for the rat. Further studies are needed to examine this issue by measuring horizontal and vertical trabeculae, separately.

Analysis of the skeletal changes in rat long bone metaphysis has always been difficult due to continued bone elongation during the first third of the rat's lifespan. The situation is even worse when the tested agent has an effect on the bone growth. Hansson *et al.* [10] investigated the normal longitudinal growth rate at proximal tibia in female rats. Results showed that the rate is very high in 20 days old rats (356 μm/d), but it rapidly decreases to 46 μm/d when the rats are 100 days old. The current study demonstrates that in female rats over 3 months of age, the proximal tibial longitudinal growth rate continues to decrease rapidly to 10 μm/d when rats are 7 months old. Thereafter, the rate slowly decreases to 2.4 μm/d in rats at 12 months of age. In some rats over 12 months, the growth plate in the proximal tibia is partially closed and replaced by calcified bone. These findings are in agreement with those of Wronski *et al.* [42], in which he and colleagues reported the longitudinal growth rate of sham ovariectomized control rats aged 3 to 21 months. With such normative data now available, one can select a suitable age of rats with an appropriate longitudinal growth rate to meet the needs of one's experimental purpose. The young rat is an excellent animal model for evaluating effects of testing agents on new bone growth. The older rat is suitable for studies of prevention of bone loss and maintenance of bone mass where bone elongation would complicate the analysis.

Doubts have been raised when the rat long bone metaphysis is used as a model to simulate human cancellous bone remodeling [7]. Whether or not bone remodeling similar to that of humans will occur in rat tibial metaphyseal cancellous bone is still an open question. Human cancellous bone metabolism is regulated by two mechanisms: mini-modeling and remodeling [9, 12]. Mini-modeling drifts trabeculae by removing old bone from one surface and adding new bone on the opposite surface, while remodeling simply renews the bone mass of trabeculae by removing a packet of primary or old lamellar bone then refilling the lacuna with a packet of new lamellar bone. These two mechanisms are responsible for the development and maintenance of cancellous bone mass and architecture throughout the life span. Mini-modeling dominates before skeletal maturity while remodeling dominates after. During growth, the remodeling process is responsible for the replacement of primary bone by lamellar bone. This same process occurs in rat proximal tibial metaphysis. Kimmel and Jee [20] reported that in the primary spongiosa the calcified cartilage area rapidly decreased when lamellar bone area increased with increasing tissue age. More recently, studies in our laboratory [15, 16] demonstrate evidence of sequential basic multicellular unit (BMU) based cancellous bone remodeling in rat proximal tibial metaphysis, which is similar to that described in humans [6], indicating that human-like bone remodeling does occur in the long bone metaphysis after a rat reaches the age when very little longitudinal growth occurs.

In the vertebral bodies, longitudinal growth rate is much slower than that in long bones and the growth plate closes earlier [4, 23, 24]. Their short growth period allows the use of younger animal models for studies to mimic human-like cancellous bone remodeling activity in long bone metaphysis. BMU based bone remodeling in the rat is observed in the secondary spongiosa of caudal [4] and lumbar [20] vertebral bodies. However, we should be aware that cancellous bone in vertebral bodies is less sensitive in response to the testing agents than that in long bones [23, 24].

Between 3 and 16-months of age, the proximal tibial metaphysis lost 40% of its cancellous bone, reduced the trabecular width by 5%, lowered trabecular number by 37%, and increased trabecular separation by

Table 5. Age related changes on tibial shaft cortical bone histomorphometry.† See Table 2a and 2b for definitions of the abbreviations.

Age (mos.)	T.Ar (mm^2)	Ct.Ar (mm^2)	Ma.Ar (mm^2)	% Ct.Ar (%)	% Ma.Ar (%)	Periosteal Surface			Endosteal Surface		
						L.Pm (%)	MAR (μm/d)	BFR (*)	L.Pm (%)	MAR (μm/d)	BFR (*)
K-W H test‡	0.0381	0.3070	0.0001	0.0016	0.0016	0.0002	0.0107	0.0124	0.0001	0.0104	0.0104
3	4.23	3.52	0.71	83	17	43.04	2.09	0.93	20.12	1.42	0.30
SD	0.07	0.13	0.09	3	3	23.44	0.38	0.62	8.06	0.28	0.16
7	4.48	3.66	0.82	82	18	16.76	0.60	0.13	5.99	0.21	0.02
SD	0.41	0.44	0.08	3	3	9.93	0.48	0.13	5.00	0.32	0.04
8	4.57	3.73	0.84	81	19	13.52	0.31	0.05	5.29	0.16	0.01
SD	0.39	0.38	0.10	2	2	11.77	0.38	0.07	2.06	0.28	0.02
9	4.65	3.79	0.87	82	18	4.51	0.22	0.01	4.18	0.00	0.00
SD	0.33	0.22	0.14	2	2	1.38	0.02	0.00	0.91	0.00	0.00
10	4.75	3.83	0.93	81	19	4.38	0.23	0.01	3.68	0.00	0.00
SD	0.29	0.20	0.13	2	2	1.41	0.06	0.00	1.41	0.00	0.00
12	4.82	3.88	0.95	81	20	4.27	0.23	0.01	3.64	0.00	0.00
SD	0.21	0.19	0.05	1	1	1.44	0.06	0.00	0.88	0.00	0.00
14	4.81	3.77	1.04	78	22	4.18	0.23	0.01	1.80	0.00	0.00
SD	0.28	0.35	0.10	3	3	1.62	0.05	0.00	0.74	0.00	0.00
16	4.87	3.75	1.12	77	23	4.11	0.23	0.01	1.68	0.00	0.00
SD	0.24	0.19	0.08	1	1	1.07	0.04	0.00	1.01	0.00	0.00
Linear regression analysis (age as independent parameter)											
Inter-cept	4.166	3.568	0.598	85.275	14.725	37.438	1.628	0.685	17.482	1.073	0.213
Slope	0.050	0.019	0.031	-0.465	0.465	-2.821	-0.122	-0.059	-1.256	-0.093	-0.019
SE	0.011	0.010	0.004	0.076	0.076	0.423	0.017	0.010	0.159	0.012	0.003
r	0.520	0.254	0.734	0.617	0.617	0.649	0.679	0.597	0.711	0.694	0.619
p	0.0001	ns	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

† Six rats in each group. ‡ Kruskal-Wallis H-test * in $\mu m^2/\mu m/d$

78%. On the contrary, in the same period, the lumbar vertebral body lost no cancellous bone, gained 35% in trabecular width dropped 30% in trabecular number and increased trabecular separation by 40%. The difference in behavior of these sites can be partially attributed to a factor of two slower turnover rates of the lumbar vertebral body. However, there was sufficient time for the lumbar vertebral body to lose some bone in the 9-month interval because the bulk of proximal tibial metaphyseal cancellous bone loss occurred in 6 months. Therefore, other factors must be in play such as differences in regional adaptation to mechanical usage [9, 26].

Accurately estimating the timing of the bone remodeling cycle is important for designing a drug testing experimental period. It was suggested by Frost [8] and Parfitt [25] that to evaluate the true effects of a testing agent on bone, the treatment period should be longer than the remodeling period [9]. In the current study, the average life span of each remodeling cycle in female Sprague-Dawley rats is approximately 24 days. It is very short when compared with 100 days for dog [41], a large animal model. The advantages of using the rat as an animal model (in terms of saving time and labor expenses for housing the animal) are clearly demonstrated after comparing lengths of the experimental periods for different animal models.

The data gathered from the rat diaphyseal cortical bone shows a similar pattern to that of a human study by Ruff and Hayes [33]. There is a continued expansion in both total cross section area and marrow cavity with aging from the cumulative effects of periosteal bone formation and endocortical bone resorption. At 3 months of

age, approximately 43% of periosteal surface and 20% of endocortical surface are actively forming new bone. In the following 5 months the formation activity decreases rapidly. At 9 months of age, only 4% of the formation surface with a very low apposition rate is observed on the periosteal surface, and only a single label on the endocortical surface. A small rate of periosteal bone formation continues for up to 16 months of age while the endocortical bone formation almost completely stops after 9 months of age. However, as indicated in Table 5, the cortical area does not change with increasing age, indicating that up to 16 months of age, the amount of newly formed subperiosteal bone is equivalent to that of endocortical resorbed bone. In other words, after maturation, the age-related cortical bone change is characterized by a redistribution of equal amounts of bone mass from the endocortical surface to the periosteal surface. The same conclusion could be drawn from mid-femoral shaft cortical bone changes in Sontag's study [39], where he found cortical bone volume unchanged while marrow volume continued to increase between 100 days and 29 months of age.

Subperiosteal expansion more than offsets the mechanical effects of age-related endocortical bone loss because it is located so as to best withstand bending and torsional stresses applied to the diaphysis. The tendency to continually increase subperiosteal area, and thus presumably the polar moments of inertia in older adult rats, is qualitatively consistent with the data presented by Jee *et al.* [13]. The tendency to lose endocortical bone with age and yet maintain or enhance the structural-mechanical strength of the diaphysis is also observed in human long bones [5, 22, 30, 31, 32]. Up to 16 months of age, no indication of age-related osteopenia could be observed in mid-tibial diaphyseal cortical bone of the rat. This may be due simply to the lack of intracortical bone remodeling in rats. Whether intracortical bone loss will occur in rats older than 16 months remains to be investigated. Nevertheless, rat cortical bone is an excellent model for studying the effects of various agents on bone modeling. Recently, it is employed as a successful model in a series of experiments, by Li and Jee [21] and Jee *et al.* [13], to evaluate adaptive cortical bone changes under either an underloaded or a mildly overloaded environment.

In summary, with many similar skeletal characteristics to humans, the rat is an appropriate small animal model for skeletal biological studies, as long as the age, skeletal site, length of the experiment and histomorphometric measurements are carefully selected relevant to the type of scientific question being asked. Since in the rat bone remodeling occurs principally in cancellous bone and modeling occurs in cortical bone, the greatest value in using the model is that it allows us to study these two different skeletal metabolic activities - to some degree - as separate phenomena in the same animal, under the same physiologic and biomechanical circumstances.

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Discussion with Reviewers

M.W. Lundy: Why is vertebral bone less sensitive when the mineralization rate is similar in vertebral and metaphyseal bone. One would think that an increase in bone formation would be easier to observe when the rate is initially low, assuming precursor cells are available.
Authors: We really do not know and can only speculate. One factor may be the difference in the mechanical usage of the spine versus a long bone. Also, one should not compare the data of two cancellous bones from these bones. In the proximal tibial metaphysis, we analyzed the secondary spongiosa only, while in the lumbar vertebral, we included the entire spongiosa made up of two metaphyses and a central portion connecting the two. In the future we will separate the parts. Finally, we do not think you can assume precursor cells are equally available or there is an endless supply on call in a bone. Possibly there are fewer available precursor cells in the lumbar vertebral body that makes it more insensitive. We have found that the corticoendosteal surface in the tibial midshaft free of trabecular bone to be more responsive to prostaglandin E_2 (PGE_2) than long bone and vertebral metaphyseal cancellous bone sites. Presumably there is an endless supply of non-competing precursor cells lining the intracortical vascular channels while the supply is more limited in the metaphyses.

It is not true in our experience that "an increase in bone formation would be easier to observe when the rate is initially low". We have found that PGE_2 treated proximal tibial metaphysis induce 4 times more bone than in the lumbar vertebral body in the same 7-month-old rats [23, 24]. In Tables 3 and 4, you will find that the bone formation rate in the proximal tibial metaphysis is twice that in the lumbar vertebral body.

M.W. Lundy: The osteoid perimeter is less in the older rats than the labeled perimeter. What is the osteoid perimeter in the metaphyseal bone? Why was osteoid width not estimated? If osteoid width is too narrow to measure, how can osteoid perimeter be accurately determined in response to treatment? Anything that increases matrix apposition rate would be expected to increase osteoid width, making the osteoid perimeter easier to measure, artifactually increasing osteoid perimeter.
Authors: We did not measure osteoid perimeter in the proximal tibial metaphysis nor did we determine osteoid width. We will do it in the future.

R.B. Martin: You discuss the occurrence of mini-modeling versus remodeling. Sites where sequential episodes of resorptive and formative mini-modeling occur could easily be confused, semantically as well as histologically, with remodeling. Please discuss this problem. How much time must pass between the two activities before they are no longer considered to be coupled?. This seems to be a difficult problem in definitions because reversal times in human osteoporosis can be several months long. While most modeling and mini-modeling may involve drifts in one direction, so that resorption and formation should not occur on the same surfaces, it is by no means certain that this is almost always the case. The endosteal surfaces of bones like the monkey femur suggest drifts frequently go first one direction and then the other. How should we handle this problem?
Authors: We are well aware of the problems you mention, but we do not have any bright ideas on how to tackle them. We have a difficult time deciding between a growth arrest and a reversal cement line in the rats. If it is possible to differentiate between such cement line, it may be possible to answer the questions you posed. It would be a great thesis problem for a graduate student.

My (W.S.S. Jee) current bias is that modeling or mini-modeling (formation and resorption drift), involves activation followed by formation (A → F), and activation followed by resorption coupled to reduced formation (A → R → F↓). Activation followed by resorption (A → R) can only occur where bone surfaces are unavailable for bone formation. In a resorption drift, bone resorption exceeds formation. Activation of resorption appears to be always coupled to formation. This is seen in the case of drifting alveolar bone (Vignery, Baron *et al.*, Anat. Rec. **196**: 191-202) where resorption was coupled to depressed formation. This is the same situation in what you describe for your monkey femur. Thus, formation drift is accomplished by activation followed by formation (A → F) and the classical example is formed at the periosteal surface. Or formation drift can be achieved by remodeling (A → R → F↑). And resorption drift is accomplished by activation followed by resorption coupled to depressed formation or uncoupled formation for lack of surface for formation (A → R → F↓ or A → R).

R.G. Erben: The increase in wall width with age observed in this rat study is at variance with the consistent finding of an age-related decline in wall width in humans. Moreover, the precipitous fall in mineral apposition rate between 3 and 7 months of age reported in vertebral cancellous bone in the present study is in disagreement with the study by Wronski *et al.* [42] which reports only a mild decrement in mineral apposition rate between 3 and 7 months of age in sham-ovariectomized Sprague-Dawley rats.
Authors: We can only speculate on why we have an increased wall thickness between 3 and 16 months. Possibly there was an increase in resorption depth in each remodeling site. We do not believe there is a real variance with the age-related decline in wall width in humans. In humans you are dealing with an adult skeleton, while we are dealing with a slowly growing bone. In our rats, we are arriving at what one would call a peak thickness in wall width. No one has studied wall width in older rats, but I (WSSJ) postulate that the wall width will decline as in man.

We do not know why we disagree with the report of Wronski *et al.* It may be that we were determining

wall width from modeling and remodeling sites. We hesitate to compare studies until we exchange material and standardize our two methods of analysis.

R.G. Erben: With formation periods between about 7 and 20 days in vertebral cancellous bone (Table 4), the authors should be aware that a marker interval (between fluorochrome double labeling) of 10 days will result in a considerable label escape error, as a consequence, in an underestimation of bone formation rates. At 3 months of age, the marker interval (10 d), is even longer than the formation period (6.89 d). Therefore, from a theoretical point of view, no fluorochrome double labels should have appeared in the vertebral cancellous bone of rats in this age group. However, the authors report high values for labeled perimeter and bone formation rate in the vertebrae of 3-month-old rats (Table 4). A possible explanation for this obvious discrepancy is the hypothesis that in the rat vertebral cancellous bone both modeling and remodeling occur at the same time. This would also explain the high values for mineral apposition rate in young animals, in which modeling activities might be predominant. Please comment.

Authors: You are correct. As mentioned in the discussion above with R.B. Martin, we admit we could not distinguish between growth arrest and reversal cement lines so we must have a mixture of modeling and remodeling sites.

It was unfortunate we did not take into consideration the label escape error, so readers beware that these reported values are for an interlabeling interval of 10 days.